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Reducing Interferences in Glycosylation Site Mapping

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ABSTRACT

A current method to locate sites of *N*-linked glycosylation on a protein involves the identification of deamidated sites after releasing the glycans with peptide-*N*-glycosidase F (PNGase F). PNGase F deglycosylation converts glycosylated Asn residues into Asp. The 1-Da mass tag created by this process is observable by liquid chromatography–tandem mass spectrometry analysis. A potential interference to this method of *N*-glycosylation site mapping is the chemical deamidation of Asn residues, which occurs spontaneously and can result in false positives. Deamidation is a pH-dependent process that results in the formation of iso-Asp (i-Asp) and native Asp (n-Asp) by a succinimide intermediate, whereas PNGase F deglycosylation results in the conversion of the glycosylation Asn residue into n-Asp. *N*-linked glycosylation sites can thus be identified by the presence of a single chromatographic peak corresponding to an n-Asp residue within the consensus sequence Asn-X-Ser/Thr, whereas sites of deamidation led to 2 chromatographic peaks resulting from the presence of n-Asp and i-Asp. The intent of this study is to alert investigators in the field to the potential and unexpected errors resulting from this phenomenon and to suggest a strategy to overcome this pitfall and limit the number of false-positive identifications.

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INTRODUCTION

Glycosylation is an important and highly regulated mechanism of secondary protein processing within cells, playing a vital role in determining protein structure, function, and stability. *N*-linked glycosylation of proteins occurs on Asn residues by an *N*-glycosidic bond. In eukaryotic cells, *N*-linked glycosylation occurs in a consensus sequence Asn-X-Ser/Thr, in which X can be any amino acid except proline.[1] Changes in *N*-linked glycosylation have been associated with different diseases, including rheumatoid arthritis, type 1 diabetes, Crohn’s disease, and cancers,[2],[3],[4],[5],[6]

indicating the importance for the accurate analytical characterization of this post-translational modification.

Chemical deamidation of Asn residues is a well-known and well-studied post-translational modification in proteins.[7],[8] It occurs spontaneously on proteins and peptides and influences several biological processes. Deamidation has been reported to be related to Alzheimer's disease and cataracts,[9],[10],[11] and it has also been thought to function as a "molecular clock" in aging.[12],[13] Nonenzymatic deamidation of Asn results in the formation of native Asp (n-Asp). Under mildly alkaline conditions, Asn deamidation happens mainly through the formation of a succinimide ring intermediate that is quickly hydrolyzed to n-Asp and iso-Asp (i-Asp), with i-Asp predominating in a 1:3 ratio.[8],[14],[15] This reaction is reversible and occurs at pH >5 via a base-catalyzed pathway. The deamidation rate can be influenced by several factors related to the protein sequence and environmental changes, such as temperature, ionic strength, and pH.[16] It has been shown that deamidation of Asn followed by Gly or Ser is predominant because of the small and hydrophilic side chains of these amino acids.[17],[18],[19] The deamidation rate of peptides increases significantly under conditions typical of tryptic digestion, that is, incubation in digestion buffers at pH 8 for 8–16 hours at 37 °C.[20] Consequently, the conditions used for sample preparation can induce peptide deamidation that is not present in the initial sample.

Identification of deamidated sites by liquid chromatography–tandem mass spectrometry (LC/MS/MS) is a step in the assignment of *N*-glycosylation sites after peptide-*N*-glycosidase F (PNGase F) deglycosylation. Release of *N*-linked glycans is commonly done using a glycosidase such as PNGase F.[21] Peptide-*N*-glycosidases convert glycosylated Asn residues into Asp, resulting in a mass shift of 1 Da at the site of modification. This leads researchers to assign potential sites of *N*-linked glycosylation based on the identification of Asp within the consensus sequence Asn-X-Ser/Thr. Mechanistically, the enzymatic deglycosylation will only lead to n-Asp, not the mixture of n-Asp/i-Asp produced by chemical deamidation. However, this is not conclusive for assigning potential sites of *N*-linked glycosylation because both naturally occurring and experimentally induced deamidation of Asn may convert Asn into n-Asp, resulting in the same 1-Da mass shift.[22]

Attempts in our laboratory to map sites of *N*-linked glycosylation on human epidermal growth factor receptor 2 (HER2) employing PNGase F deglycosylation followed by LC/MS/MS produced puzzling results. Specifically, multiple sites of Asn deamidation

were identified, but none of these were in the Asn-X-Ser/Thr consensus sequence. Both naturally occurring and experimentally induced deamidation of Asn into n-Asp results in a 1-Da mass shift, allowing for possible interferences resulting in false positives. This prompted us to investigate the possibility of deamidated peptides being misidentified as sites of *N*-linked glycosylation during PNGase F deglycosylation and to develop a useful approach to differentiate between chemical and enzymatic deamidation.

MATERIALS AND METHODS

Peptide digestion

Adalimumab and a glycoprotein consisting of the extracellular domain of HER2 fused with the Fc domain of human IgG1 from GlycoScientific (Athens, GA, USA) were first buffer exchanged with 50 mM ammonium bicarbonate (pH 7.8) or 50 mM ammonium acetate (pH 6.8). They were then reduced using 200 mM DTT and alkylated using 1 M iodoacetamide, both purchased from Sigma Aldrich (St. Louis, MO, USA), to a final concentration of 5 mM DTT and 8 mM iodoacetamide. Sequencing grade trypsin purchased from Promega (San Luis Obispo, CA, USA) was added at 20:1 (w/w, protein/trypsin) for incubation at 37 °C. After 48 hours passed, the samples were removed and divided in half. One half of the sample was dried down and resuspended in 80% acetonitrile and 20% H_2O. Five hundred units of PNGase F were used to deglycosylate the other half of the sample and incubated for an additional 48 hours at 37 °C. After a total of 96 hours passed, the sample was removed from the incubator and set on a 100 °C heat block for 5 minutes to inactivate the PNGase F. The sample was then dried down and resuspended in 80% acetonitrile and 20% H_2O.

LC/MS/MS settings and instrumentation

Data were acquired using a 4000 Q Trap (AB Science, Chatham, NJ, USA) with a Nexera UFLC (Shimadzu, Columbia, MD, USA). Peptides were separated using a 2.1-mm × 150-mm HALO Penta-HILIC column packed with 2.7-um diameter superficially porous particles that have a 90-Å pore diameter (Advanced Materials Technology, Wilmington, DE, USA) at 60 °C. The mobile phases used in the separation were 0.1% formic acid, 50 mM ammonium formate in water (A), and 0.1% formic acid in acetonitrile (B). The peptides were bound to the column in 82% B, and a linear gradient to 54% B over 30 minutes was initiated to elute the peptides. Skyline was used to make a transition list.[23]

RESULTS AND DISCUSSION

Recent attempts in our laboratory to map sites of *N*-linked glycosylation on HER2 protein in breast cancer employing PNGase F deglycosylation followed by LC/MS/MS produced puzzling and ambiguous results. In particular, multiple sites of deamidation, that is, sites of *N*-glycosylation, were identified but not found within the Asn-X-Ser/Thr consensus sequence required for *N*-linked glycosylation.[1] Our interest was in determining whether the analytical approach was defining accurate sites of modification. It was hypothesized that chemical deamidation could explain the deamidation observed because deamidation is known to occur at pH 7.8, a common pH for digestion with trypsin and PNGase F. Ambiguities led us to examine the HER2 data in greater detail. The potential for chemical deamidation occurring during deglycosylation was investigated using hydrophilic interaction liquid chromatography (HILIC)–mass spectrometry to analyze trypsin/PNGase F digested adalimumab.

Deamidation of Asn increases the hydrophilicity of the altered residue, and HILIC polar stationary phases have the potential to separate these changes in hydrophilicity while allowing for quantitation of unmodified species and their modified counterparts.[24] Chromatographic analysis was thus applied to resolve enzymatic and chemical deamidation because only the later mechanism should result in a mixture of aspartyl isomers with the succinimidyl intermediate. No such mixture results from an enzymatic process, whereas chemical deamidation is expected to produce a mixture of i-Asp and n-Asp products because of the succinimide intermediate. The mechanism for chemical and enzymatic deamidation through PNGase F deglycosylation is shown in FIGURE 1. Experiments were performed in both high (pH 7.8) and low (pH 6.8) pH conditions because previous studies have shown that the deamidation rate of peptides increases significantly under conditions typical of tryptic digestion and PNGase F deglycosylation, ie, incubation in digestion buffers at pH 8 for 8–16 hours at 37 °C.[20]

The peptide EEQYNSTYR contains the 1 site of *N*-linked glycosylation in human IgG1, commonly occupied with the A2G0F glycan structure.[25] After 48 hours, the Asn version is the only species present at both high and low pH conditions (FIGURE 2A and 2B) because the presence of the glycan does not allow for deamidation to occur. PNGase was then added to remove the glycan and convert the glycosylated Asn residue to Asp, resulting in a plus–1-Da shift in the peptide's molecular weight. After the addition of PNGase F and a total incubation period of 96 hours, the representative peptide in both the high and low pH conditions continued to only show 1 peak (FIGURE 3A and 3B) corresponding to the n-Asp–containing species.

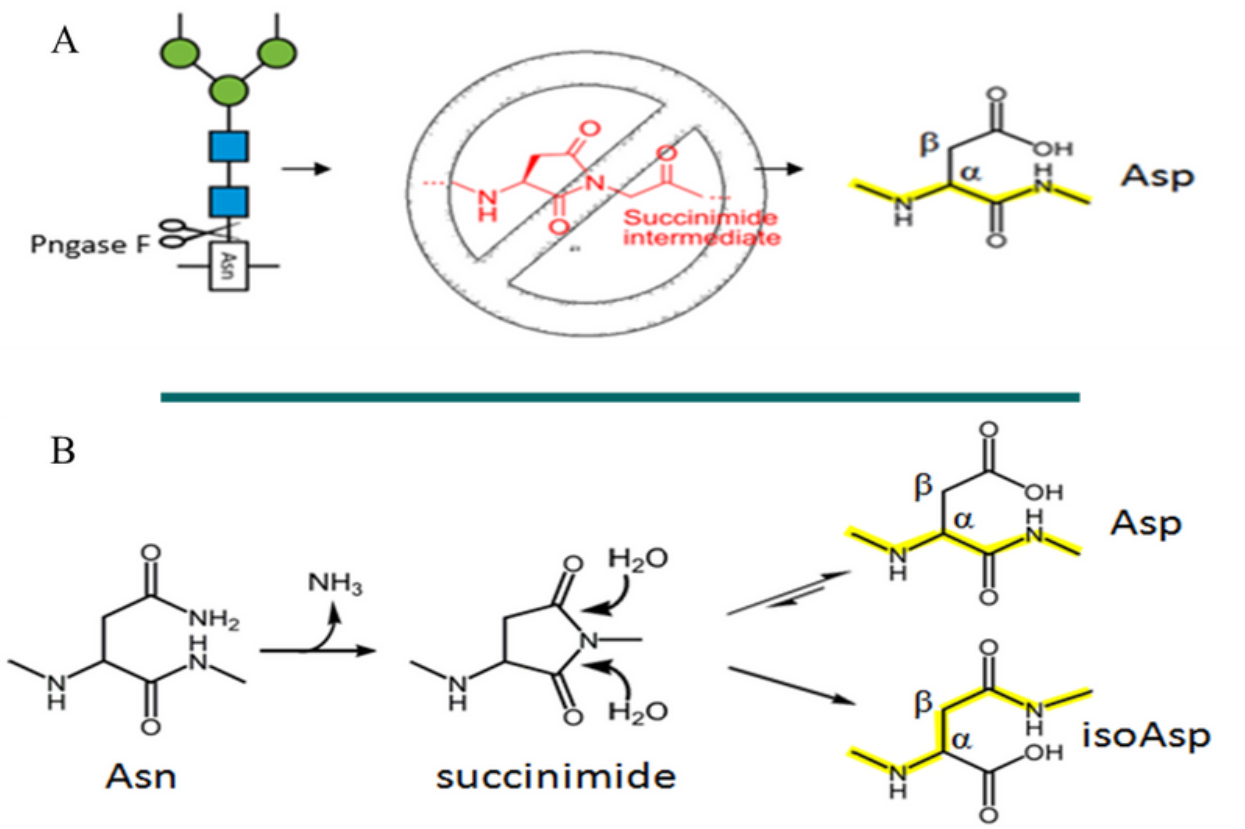

FIGURE 1

A) Mechanism of PNGase F deglycosylation. (*B*) Mechanism of Asn deamidation and Asp isomerization via a succinimide intermediate. The peptide backbone is highlighted in yellow. PNGase, peptide-*N*-glycosidase F.

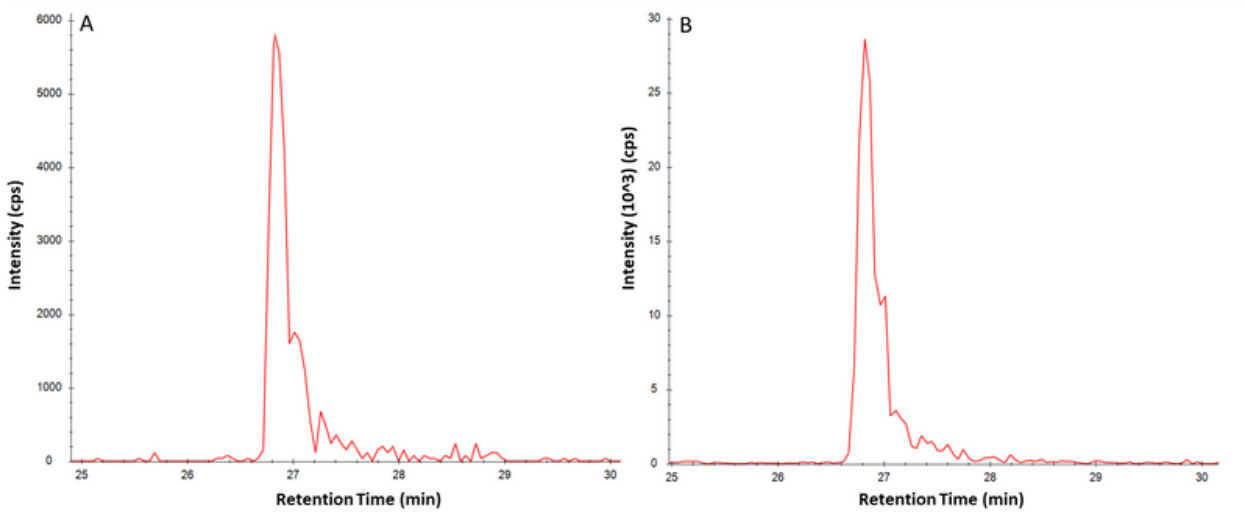

FIGURE 2
The selected reaction monitoring chromatogram of the IgG glycosylated peptide EEQYNSTYR with glycan A2G0F attached ($[1317.53]^{++}$) trypsin digested at (*A*) pH 6.8 and (*B*) pH 7.8 at 37 °C for 48 h.

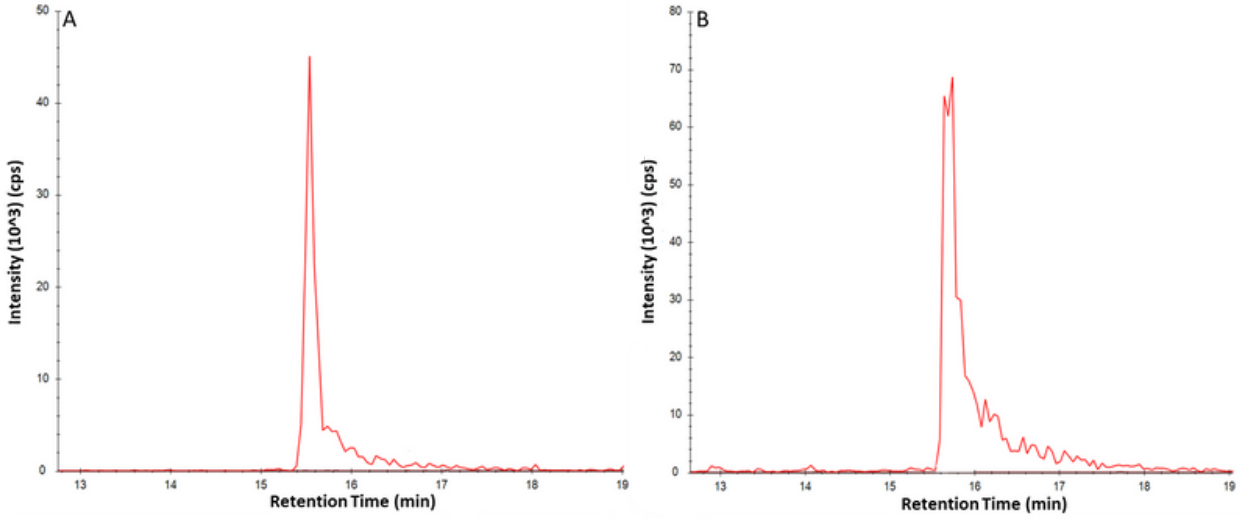

FIGURE 3
The selected reaction monitoring chromatogram of the IgG glycosylated peptide EEQYNSTYR ($[1189.51]^{+}$) after treatment with PNGase F incubating at (*A*) pH 6.8 and (*B*) pH 7.8 at 37 °C for 96 h total. PNGase F, peptide-*N*-glycosidase F.

The data demonstrate that PNGase F deglycosylation converts the Asn exclusively to an n-Asp because it does not go through a succinimide intermediate, thus leading to only 1 product. The PNGase F deglycosylation does result in an addition of 1 Da of mass to the peptide's molecular weight, but previous data shown highlight that

PNGase F deglycosylation is not the only manner leading to Asn deamidation in a sample. Deamidation is a pH-dependent process that results in the formation of 2 peaks, 1 for the n-Asp and i-Asp versions, whereas PNGase F deglycosylation results in 1 peak from the conversion of the glycosylation Asn residue into n-Asp.

The ability to differentiate sites of deglycosylation from chemical deamidation is highlighted again in the chromatogram of the representative glycopeptide (GHCWGPGPTQCVNCSQFLR) from HER2 (FIGURE 4). After 48 hours, PNGase F was added, and the sample was incubated at 37 °C for an additional 48 hours. After the addition of PNGase F, removing the glycan attached, and converting the Asn to n-Asp, only 1 peak is observed at 18.9 minutes, corresponding to the n-Asp version (FIGURE 4). The identity of the peptide was also confirmed orthogonally using an HILIC Retention prediction model.[26] The data demonstrates that PNGase F deglycosylation converts the Asn to an n-Asp and thus the i-Asp version is not observed. The single peak corresponding to n-Asp species after PNGase F treatment demonstrates that this position in HER2 is indeed glycosylated.

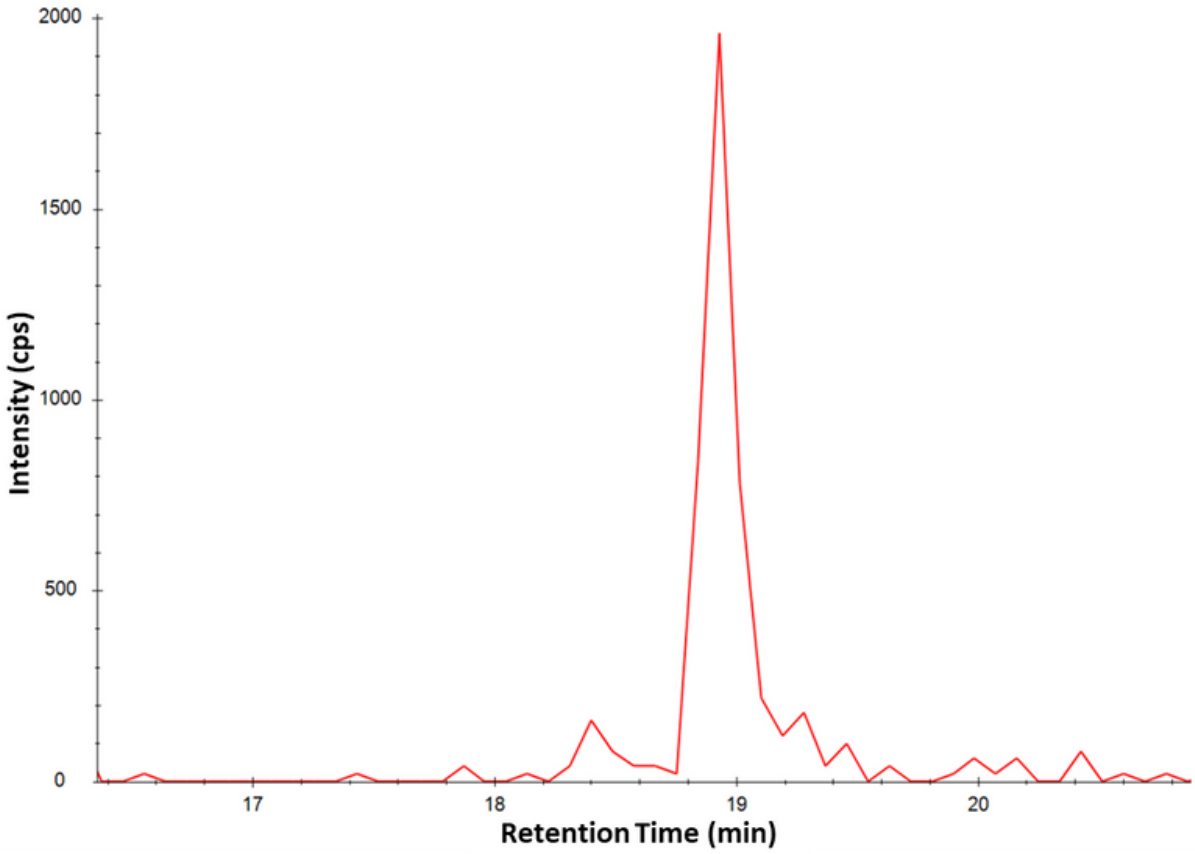

FIGURE 4

The selected reaction monitoring chromatogram of the HER2 glycosylated peptide GHCWGPGPTQCVNCSQFLR ($[1130.99]^{++}$) generated by trypsin digestion and deglycosylation with PNGase F. When combined, these 2 enzymatic digestions led to a total incubation time of 96 h at 37 °C with a pH of 7.8. HER2, human epidermal growth factor receptor 2; PNGase F, peptide-*N*-glycosidase F.

Analysis of the deglycosylated peptides led us to investigate sites of deamidation to devise a strategy to differentiate between these 2 deamidation mechanisms. The VVSVLTVLHQDWLNGK peptide from IgG1 contains an unglycosylated n-Asn in an "Asn-Gly" site, which is known to be highly susceptible to chemical deamidation. After 48 hours in pH 7.8 conditions, the formation of n-Asp and i-Asp is observed in this peptide, as shown in FIGURE 5. As determined in a previous study, n-Asp and i-Asp can be chromatographically separate through HILIC,[24] as illustrated in Figure 5. The first peak at 11.4 minutes corresponds to amidated Asn. The peak at 12.9 minutes corresponds to n-Asp, and the peak at 13.4 minutes corresponds to i-Asp. Previous studies on chemical deamidation have shown that i-Asp is 3 times more abundant than n-Asp. The ratio of peak abundances of n-Asp and i-Asp in Figure 5 is analogous to this statement.[14],[15] The least-retained peak is the unmodified form of the peptide, the

peak in the middle is the aspartyl version, and the peak most retained is the isoaspartyl version. The i-Asp version elutes later than the native form because of the hydrophilicity of the modification.[24]

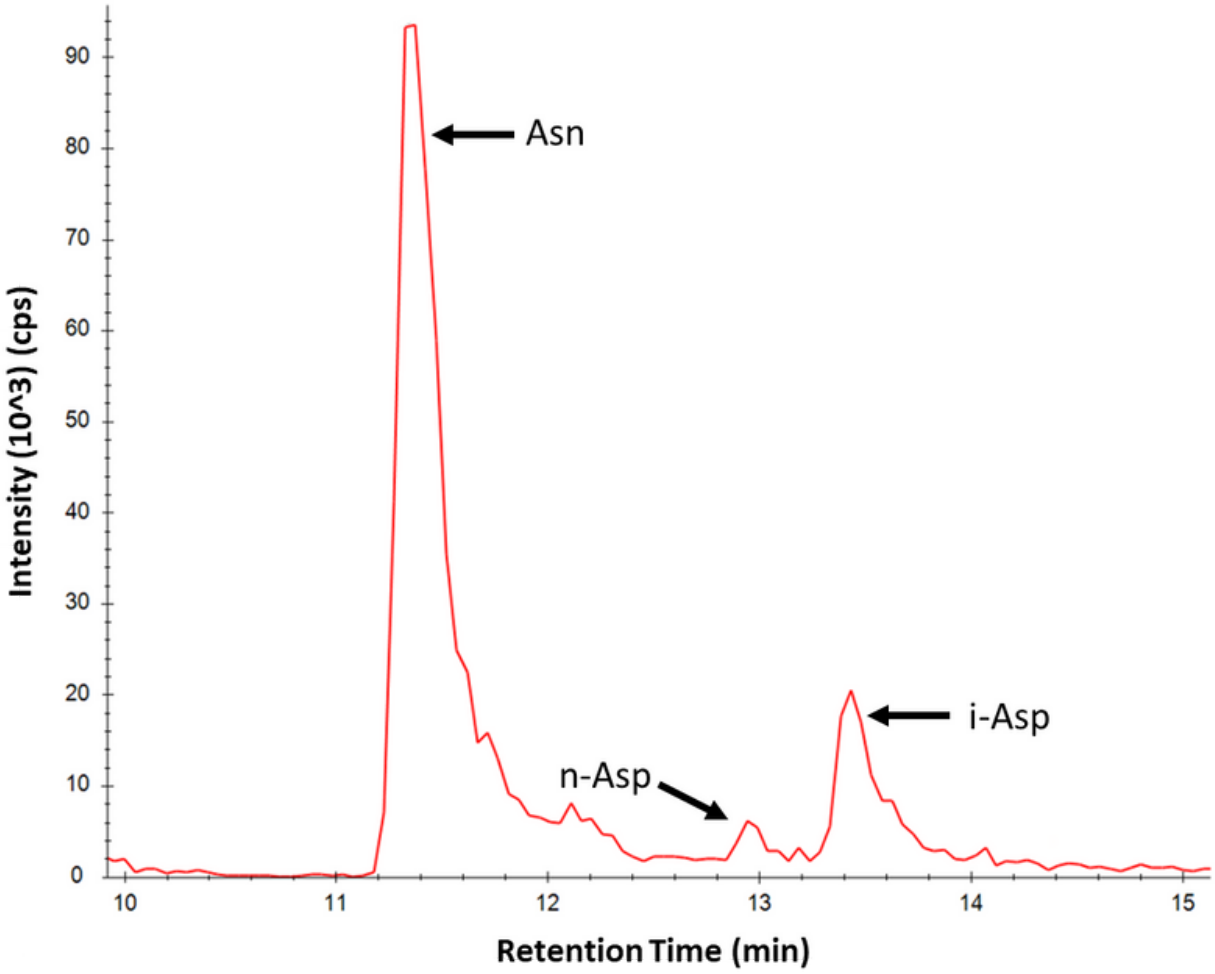

FIGURE 5

The selected reaction monitoring chromatogram of the IgG deamidated peptide VVSVLTVLHQDWLNGK ($[904.51]^{+}$), generated by trypsin digestion at pH 7.8 and 37 °C for 48 h, illustrates how the n-Asp and i-Asp versions can be separated. The peak at 11.4 min represents amidated Asn, which is also the most prominent peak in the chromatogram. The peak at 12.9 min represents n-Asp, and the peak at 13.4 min represents i-Asp. The i-Asp is 3 times the size as n-Asp. n-Asp, native Asp; i-Asp, iso-Asp.

After 48 and 96 hours in pH conditions that mimicked trypsin/PNGase F digestion, the deamidated version of this peptide eluted in 2 peaks, corresponding to the peptide containing an n-Asp or an i-Asp. These results are consistent with chemical deamidation and demonstrate that chemical deamidation occurs to a significant extent

under typical enzymatic deglycosylation conditions. The data demonstrate that deamidation is a pH-dependent process and that PNGase F deglycosylation is not the main contributing factor to the deamidation for this peptide, which indicates that the peptide is not glycosylated. The assumption that all sites of Asn deamidation resulting from enzymatic digestion/deglycosylation marks an *N*-linked glycosylation site is not consistent with the results of this experiment.

The current method of identifying *N*-linked glycosylation sites based on the presence of Asp within the consensus sequence Asn-X-Ser/Thr requires further identification of aspartyl or isoaspartyl mixtures to differentiate between chemical and enzymatic deamidation. A new method of *N*-linked glycosylation site identification through Asn deamidation that considers both chemical and enzymatic deamidation is proposed: **n**-Asp and **i**-Asp to reduce **f**alse **p**ositives (niFP).

By this proposed niFP method, *N*-linked glycosylation sites can be identified by the presence of an n-Asp residue within the consensus sequence Asn-X-Ser/Thr that also chromatographically shows the presence of 1 peak, corresponding to Asp. The site is considered to be chemically deamidated if the chromatogram shows the presence of 2 peaks corresponding to the n-Asp and i-Asp.

CONCLUSION

Naturally occurring and experimentally induced deamidation of Asn can interfere with the identification of *N*-linked sites of glycosylation. Identification of n-Asp within the consensus sequence Asn-X-Ser/Thr after deglycosylation with PNGase F is not conclusive for assigning potential sites of *N*-linked glycosylation because both chemical and enzymatic deamidation of Asn can convert Asn into n-Asp, resulting in the same 1-Da mass shift. Accurate determination of protein glycosylation sites by the current method of identification of deamidated Asn within the consensus sequence Asn-X-Ser/Thr is hindered because deamidation of Asn residues occurs both enzymatically and chemically, resulting in false positives. Chemical deamidation is a pH-dependent process that results in the formation of both n-Asp and i-Asp, which can be chromatically resolved, whereas enzymatic deamidation as seen in PNGase F deglycosylation results in 1 peak from the conversion of the glycosylation Asn residue into n-Asp. By utilizing the niFP method, *N*-linked glycosylation sites can be identified by the presence of an n-Asp residue, not a mixture of n-Asp and i-Asp, within the consensus sequence Asn-X-Ser/Thr. The site is considered to be deamidated if the chromatogram shows the presence of peaks corresponding to the n-Asp and i-Asp. The intent of this study is to alert investigators in the field to the potential and unexpected

errors resulting from this phenomenon and to suggest strategies to overcome this pitfall and prevent the identification of false positives.

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